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To: Examiner Ram Shukla
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From: Karen L. Elbing, Ph.D.

Re: U.S.S.N.: 08/908,453
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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gary Ruvkun et al.	Art Unit:	1632
Serial No.:	08/908,453	Examiner:	R. Shukla
Filed:	August 7, 1997	Customer No.:	21559
Title:	AGE-1 POLYPEPTIDES AND RELATED MOLECULES AND METHODS		

Assistant Commissioner For Patents
Washington, DC 20231

SUPPLEMENTAL REPLY TO EXAMINER'S ACTION

Applicants thank the Examiner for the interview granted on February 21, 2002.

As discussed in that interview, Applicants submit herewith a Declaration from Dr. Gary Ruvkun, describing additional experimental data provided during the interview. In addition, Applicants summarize below evidence made of record to date in this case, indicating that Applicants' newly discovered AGE-1 nucleic acid encodes a polypeptide having PI-3 kinase activity.

As discussed during the interview, AGE-1's identification as a PI 3-kinase is based on the following evidence: (i) sequence homology and characteristic structural motifs, (ii) physical interactions, (iii) genetic interactions, and (iv) biochemical evidence. Each is discussed in more detail below.

AGE-1 Sequence Homology and Characteristic Structural Motifs

As noted previously by Applicants, *age-1* shares sequence homology with other PI 3-kinases. *C. elegans* AGE-1 is the worm protein most homologous to known PI 3-

kinases from mammals, and AGE-1 displays structural motifs characteristic of PI 3-kinases, including a p85 interaction domain and a lipid kinase domain. The closest mammalian homolog of *C. elegans* AGE-1 is mammalian p110 PI 3-kinase. When the p110 sequence was used to search the worm proteome database, AGE-1 was found to be p110's closest homolog. The random probability of alignment of AGE-1 with mammalian p110 kinase was extremely low, less than e^{-100}. Moreover, when the same search was conducted using a mammalian P110 PI-3 kinase query (for example, XP_066258, phosphoinositide 3-hydroxykinase p110-alpha subunit *Homo sapiens*) the next closest sequence hit in the *C. elegans* proteome database was 30-logs lower in probability, an enormous step down in sequence alignment terms. In addition, when the *C. elegans* AGE-1 sequence was used to search a mammalian proteome database, mammalian PI 3-kinases were also found to be AGE-1's closest homologs. Again, the random probability of this alignment to occur by chance rather than to reflect true orthology was extremely low, less than e^{-98}. These results provide strong evidence that *age-1* is the *C. elegans* ortholog of biochemically characterized mammalian PI 3-kinases.

Also consistent with these high levels of sequence similarity, Applicants have found that substitution of an amino acid conserved between the AGE-1 polypeptide and mammalian p110 PI 3-kinase leads to complete loss of AGE-1 activity. This result lends further credence to the biological relevance of the sequence shared between AGE-1 and mammalian p110.

AGE-1 Physical Interactions

As a PI 3-kinase, AGE-1 would be expected to exhibit the characteristic physical interactions of a PI 3-kinase. PI 3-kinases are heterodimeric enzymes that consist of a catalytic subunit, p110, and an SH2-domain-containing adapter subunit. In vertebrates p110 interacts with p85 or p55, SH2-domain-containing adapter subunits, through its amino-terminal domain; this interaction is required to activate the catalytic activity of p110. Examining the AGE-1 sequence, the amino-terminal domain of AGE-1 and p110 are 25% identical, suggesting that this interaction domain is under selective pressure to remain the same, perhaps due to the presence of a common regulatory partner, such as p85 or p55.

Applicants used a BLAST search of the *C. elegans* genome to identify p85 or p55 homologs. This search identified a single *C. elegans* polynucleotide, *y110a7a-2.k*, that shared significant sequence homology (random probability of alignment: 3.8×10^{-29}) with mammalian p85 and p55. *y110a7a-2.k* encodes a p55-like adapter subunit, and was subsequently renamed *aap-1* for age-1 adapter protein-1. Sequence comparisons using BLAST and PILEUP algorithms (Genetics Computer Group, WI) showed that the structure of AAP-1 was most closely related to SH2 domains from other Class IA PI 3-kinase adapter subunits. Based on the mammalian PI 3-kinase interactions, Applicants predicted that the AAP-1 PI 3-kinase adapter subunit would bind to the amino-terminal domain of AGE-1.

Applicants tested this prediction by producing recombinant AAP-1 and assaying for AAP-1 binding to the amino-terminal domain of AGE-1. AGE-1 (amino acids 1-268)

was efficiently co-precipitated by AAP-1-containing beads; AGE-1 failed to bind to beads lacking AAP-1. These results indicated that AGE-1 specifically interacts with AAP-1, and confirmed Applicants' prediction that the interaction between mammalian p110 and p85 is conserved in their *C. elegans* counterparts, AGE-1 and AAP-1. AGE-1's interaction with AAP-1 also provides support for AGE-1's identification as a PI 3-kinase, because of its shared physical interaction with SH2 adapter proteins.

This physical interaction was further demonstrated by the following *in vivo* experiment. If *aap-1* encodes the authentic regulatory subunit for AGE-1 PI 3-kinase, then *aap-1* gene function should be required for *age-1* function *in vivo*. To test this prediction, RNA-mediated interference was used to reduce *aap-1* gene function. Specifically, in *C. elegans,* injection of double-stranded RNA of a target gene results in RNA-mediated interference with target gene expression. This interference effectively reduces or eliminates the gene's activity in the injected animal and its progeny.

In Applicants' experiment, RNAi was used to test the prediction that animals with decreased *aap-1* function would resemble *age-1* loss-of-function mutants, *i.e.,* display constitutive arrest at the dauer larval stage. These studies were carried out in a sensitized genetic background that allowed the detection of small decrements in *aap-1* activity. Applicants found that, in a sensitized background, *aap-1* RNAi strongly enhanced dauer arrest (compared to that observed for uninjected control animals), as expected if *aap-1* gene function is required for *age-1* function *in vivo*.

This physical and genetic evidence indicates that the SH2-domain-containing adapter protein, AAP-1, interacts with AGE-1, consistent with the interaction of their mammalian homologs and consistent with the identification of AGE-1 as a PI 3-kinase.

AGE-1 Genetic Interactions

Additional evidence that AGE-1 is a PI 3-kinase is provided by genetic interactions that place AGE-1 in the insulin pathway. Vertebrate PI 3-kinases function in insulin signaling. If AGE-1 functions as a PI 3-kinase, then by analogy to mammalian systems AGE-1 would be predicted to act downstream of the *C. elegans* insulin receptor tyrosine kinase, which has specific phosphotyrosine motifs (YXXM) associated with p85 SH2-domain binding. Applicants tested this prediction genetically and found that, not only does AGE-1 function downstream of the *C. elegans* insulin receptor, but AGE-1 also functions upstream of PDK and AKT kinases. This is relevant because PDK and AKT kinases have pleckstrin homology domains that are specifically regulated by the product of AGE-1, that is, PIP3.

Moreover, Applicants' genetic studies with *daf-18*, another component of the insulin-like signaling pathway, provide further support for AGE-1's identification as a PI 3-kinase. *daf-18*, the *C. elegans* homolog of mammalian PTEN, encodes a lipid phosphatase that dephosphorylates phosphoinositides *in vitro* and lowers PIP3 levels *in vivo* by inhibiting PIP3 accumulation in response

to insulin signaling. Since PTEN dephosphorylates PIP3, DAF-18 may normally function to decrease the PIP3 output of AGE-1 PI 3-kinase signaling. If so, mutations in *daf-18* should suppress mutations in *age-1*. In Applicants' experiments, this hypothesis was found to be correct; Applicants determined that mutations in *daf-18* suppressed mutations in *age-1*. Moreover, Applicants found that loss of DAF-18 enhanced PIP3 signaling to AKT kinases, consistent with AGE-1 generating a PIP3 second messenger and again consistent with AGE-1's role as a PI 3-kinase.

AGE-1 Biochemical Evidence

Finally, as biochemical evidence that AGE-1 is a PI 3-kinase, Applicants again direct the Examiner's attention to the previously submitted publication by Babar et al. (*Neurobiology of Aging* 20:513, 1999). In this reference, the authors treated *C. elegans* with a known chemical inhibitor of mammalian PI 3-kinases, a chemical termed LY294002. This treatment mimicked the effects of AGE-1 mutations (pages 516-517), as measured by dauer formation, thermotolerance, and life span. This experiment indicates that the *in vivo* outcome of a loss of AGE-1 function parallels the *in vivo* outcome of a loss of PI 3-kinase activity. This biochemical result is therefore consistent with AGE-1 functioning as a PI 3-kinase.

Acceptance by Experts of AGE-1's Role as a PI 3-Kinase

As further evidence that AGE-1 is a PI 3-kinase, and is so accepted by experts, Applicants submit herewith the following references: Guarente et al. (*Nature* 408:255, 2000) and Vanhaesebroeck et al. (*TIBS* 22:267, 1997). Each of these references was written by a third party expert, and each accepts that AGE-1 is a PI 3-kinase.

Taking each in turn, Guarente provides a review of the genetic pathways that regulate ageing in model organisms, particularly *C. elegans*. Guarente discusses the insulin-like signaling pathway that regulates the lifespan of *C. elegans*. With respect to Applicants' work, at page 257, first column, last paragraph, Guarente states:

> Elegant molecular studies have shown that DAF-2 activates a conserved phosphatidylinositol-3-OH kinase (PI(3)K)/3-phosphoinositide-dependent kinase-1 (PDK1)/Akt signal transduction pathway[41-47] (Fig.3a). Reduction-of-function mutations in components of this pathway, including mutations in ***age-1*, which encodes a PI(3)K**[41], and *pdk-1*, which encodes a PDK1 homologue[43], extend lifespan.

Clearly, Guarente accepts that AGE-1 is a PI 3-kinase.

Vanhaesebroeck provides a review of PI 3-kinases in various organisms. With respect to *C. elegans*, Vanhaesebroek states:

> *C. elegans* possesses genes encoding one PI3K from each class, though only the function of the class I_A PI3K homologue has been studied to date[42]. The **class I_A PI3K gene, termed *age-1*** or *daf-23*, was identified in genetic screens for mutants that promote longevity (*age* mutants)...(Page 271, first column, lines 11-17).

Vanhaesebroeck therefore also accepts that *age-1* encodes a PI 3-kinase.

Summary

In view of the above evidence, Applicants submit that AGE-1 is a PI 3-kinase, and has been so demonstrated by a number of different criteria; first, *age-1* shares sequence homology and characteristic structural motifs with other PI 3-kinases; second, AGE-1 physically interacts with APP-1, the *C. elegans* ortholog of the mammalian PI 3-kinase-interacting protein p55; third, the function of *age-1* in the *C. elegans* insulin signaling pathway is entirely consistent with AGE-1 acting as a PI 3-kinase; fourth, genetic evidence shows that inactivation of DAF-18, a lipid phosphatase that normally lowers PIP3 levels, suppresses *age-1*; and, fifth, biochemical evidence demonstrates that treatment of worms with a PI 3-kinase inhibitor produces *in vivo* outcomes that mimic the phenotypes of worms with *age-1* mutations. In addition, the review articles submitted by Applicants show that experts both in the *C. elegans* field and in the PI 3-kinase field view AGE-1 as a PI 3-kinase.

Once more, Applicants point out that no evidence has been made of record in this case that would cause one to doubt Applicants' assertion that the AGE-1 protein has PI 3-kinase activity. And, moreover, Applicants have provided evidence from a number of sources supporting AGE-1's role as a PI 3-kinase. This basis for the enablement rejection should be withdrawn.

PI 3-Kinase Assays

In response to the Examiner's related question regarding how the activity of a *C. elegans* PI 3-kinase could be assayed, Applicants direct the Examiner to the accompanying Declaration of Dr. Gary Ruvkun, that details how such an assay could be carried out. Briefly, these assays would involve nothing more than preparation of a cell extract by disruption of the outer cuticles of worms in concentrated samples, followed by a standard PI 3-kinase assay. Methods for concentrating worms and disrupting their outer cuticles are well known in the art, as are PI 3-kinase assays, an example of which is referenced in Applicants' specification at page 35, lines 25 and 26. Alternatively, if desired, AGE-1 protein may be purified from a crude homogenate using standard methods known to the skilled artisan. The purified protein can also be used in a standard PI 3-kinase assay.

Conclusion

Applicants submit that strong evidence has been presented for the function of AGE-1 as a PI 3-kinase, and for the enabling nature of Applicants' specification. Applicants respectfully request that the enablement rejection be withdrawn.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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